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14. ABSTRACT

The incidence of renal cell carcinomas (RCCs) has risen significantly in the last 20 years. There is currently a critical unmet need for noninvasive biomarkers that can confidently predict the biological behavior of renal tumors in order to improve their accurate diagnosis and rational selection of treatment options. The goal of this pre-clinical study is to identify clinically translatable hyperpolarized 13C markers of RCC aggressiveness using established cell lines. In this work, we compared the metabolism of living immortalized cells derived from a localized renal cell carcinoma (RCC), and a metastatic RCC using hyperpolarized 13C pyruvate MR in a MR compatible bioreactor. We showed that the observed hyperpolarized pyruvate to lactate flux in these cells is likely influenced by a combination of factors including the enzyme lactate dehydrogenase (LDH), and the monocarboxylate transporters (MCT) 1 and 4 which transport pyruvate and lactate respectively. We further demonstrated that cells derived from the metastatic RCC have increased export of hyperpolarized lactate to the extracellular space compared to the cells derived from the localized RCC, and that such differences are likely mediated by the differential expression of MCT4. These results suggest that the assessment of lactate production and export using clinically translatable hyperpolarized probes has the potential to improve the noninvasive characterization of renal tumor aggressiveness.

15. SUBJECT TERMS
Renal cell carcinoma; tumor aggressiveness; Hyperpolarized 13C MR; Lactate; Pyruvate

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Introduction:

The incidence of renal cell carcinoma has been rising by 3% per year, which is largely attributed to the widespread use of crossing-sectional imaging with incidental discovery of renal tumors (1). While renal cell carcinoma can potentially be highly aggressive and metastatic, many of the incidentally discovered renal tumors are small (<4cm) and indolent cancers with low metastatic potential (2). Patients may be over-treated if all such tumors are resected. Therefore, management of small renal tumors has expanded from surgical resection to include less invasive approaches such as ablation and active surveillance. However, triage of therapies is currently difficult because of our inability to reliably determine renal tumor aggressiveness using conventional imaging. Fast hyperpolarized (HP) ^{13}C magnetic resonance spectroscopic imaging (MRSI) after injection of hyperpolarized [1-^{13}C] pyruvate substrate is a new metabolic imaging approach that takes advantage of the increased glycolysis in aggressive cancer. Increased glycolysis with high lactate production has been shown in protein studies to occur in aggressive and metastatic renal cell carcinomas (3-4). HP ^{13}C MRSI can image in real time the glycolytic flux such as the conversion of hyperpolarized pyruvate substrate to lactate, and can potentially discriminate indolent from aggressive renal cancers. In this preclinical study utilizing established RCC cell lines, we aim to test the hypothesis that HP ^{13}C MRSI after injection of HP pyruvate can differentiate between cells derived from localized and presumably indolent RCC (UMRC6 cells) and those cells derived aggressive and metastatic RCC (UOK262 cells).

Body:

Our initial MR experiment on mouse xenografts of RCCs created using established RCC cell lines revealed that the RCC xenografts created by such method had significant limitations for metabolic evaluation of the tumor. The main limitation is that there was poor perfusion of the tumor xenografts, likely a result of the increased tumor interstitial pressure in this artificially created tumor mass. The poor tumor perfusion in the xenograft is unlike the human situation where majority of the RCCs are considerably hypervascular. The poor tumor perfusion in the xenograft limits metabolic evaluation using HP ^{13}C MR. Therefore we proceeded to metabolically characterize the RCC cells using a novel MR compatible bioreactor platform. The bioreactor engineered by our group is a completely contained 3-dimensional culture system with a continuous flow of 37ºC culture medium heated by water-jacketed inlet lines. Prior to entering the bioreactor, medium is oxygenated using a Gas Exchange Module, which is filled with 95% air/5% CO_2, to preserve oxygenation, pH and provide physiologic condition (5). This platform

allows evaluation of dynamic metabolism of living cells and tissues in a physiological and controlled environment. Such bioreactor platform allows metabolic characterization of RCC cells, and discovery of clinically translatable HP ^{13}C biomarker of renal tumor aggressiveness.

Real time hyperpolarized pyruvate to lactate flux in RCC cells in the bioreactor

^{31}P spectroscopy was used to monitor changes in cell bioenergetics during the bioreactor studies. Fig. 1a shows a representative ^{31}P spectrum of UMRC6 cells during continuous perfusion in the bioreactor. NMR signals for the phosphates of the NTPs (γNTP, αNTP, and βNTP), phosphocholine (PC), inorganic phosphate (Pi), and glycerol phosphocholine are readily visible. The total NTP content was unchanged following the injection of HP ^{13}C pyruvate, indicating constant cell viability during the course of the HP experiments. Such bioreactor platform has been shown to produce highly reproducible HP MR data (5). In addition, the bioreactor also allows quantitative data analysis by normalizing the HP MR data with respect to the number of viable cells, through concomitant measurements of NTP concentration via ^{31}P MR.

After the injection of HP ^{13}C pyruvate into the bioreactor, the real time pyruvate to lactate flux was assessed for the two RCC cells lines. A representative time course of ^{13}C pyruvate and lactate in the UMRC6 cells is shown in Fig. 1b. The data were fit to a two-state model of the interconversion of pyruvate to lactate to calculate metabolic fluxes (5). The observed flux rates of pyruvate to lactate for the two RCC cell lines in the bioreactor are shown in Fig. 1c. Interestingly, the observed pyruvate to lactate flux for UOK262 cells (representative of metastatic RCC) was significantly lower than that of the UMRC6 cells (representative of localized RCC) ($P< 0.05$).

^{13}C pyruvate thermal labeling study and LDH/ MCT Expression Assays

In order to gain an understanding of the cellular processes underlying the HP pyruvate flux results, we then proceeded to: 1) perform 24 hour ^{13}C pyruvate thermal labeling study to assess the steady state pyruvate to lactate flux in the RCC cell lines; and 2) assay the mRNA expression level of LDHα, MCT1 and MCT4 in the two RCC cell lines.

The 24 hour ^{13}C pyruvate thermal labeling study showed significantly elevated steady state intra- and extra-cellular lactate level in the metastatic UOK262 cells compared to the localized UMRC6 cells (Figure 2).

LDH is the enzyme that catalyzes the conversion between pyruvate and lactate. MCT1 is the predominant transporter of pyruvate into the cells, and MCT4 is the predominant transporter

of lactate out of the cells. The mRNA expression assays showed that LDH-α was significantly higher in the UOK262 cells than the UMRC6 cells (Fig. 3), although the LDH activity was not differently between the two RCC cell types (data not shown). The mRNA expression of MCT1 was significantly higher in the UMRC6 cells than the UOK262 cells (Fig. 3). In contrast, the mRNA expression of MCT4 was significantly higher in the UOK262 cells than the UMRC6 cells (Fig. 3).

These mRNA expression data shed light on the observed HP pyruvate to lactate flux. Although the 24 hour thermal pyruvate labeling experiments showed significantly higher ^{13}C labeled lactate in the UOK262 cells compared to UMRC6 cells, the HP pyruvate MR experiments showed lower pyruvate to lactate flux in the UOK262 cells during the timeframe of the HP experiment. We postulate that such apparent discrepancy is related to the differences in MCT4 expression between the UMRC6 and UOK262 cells. The UOK262 cells have 75% higher MCT4 expression compared to the UMRC6 cells, thus allowing more rapid export of lactate out of the cells. Rapid export of lactate via MCT4 is essential for maintaining a neutral intracellular pH, and a high rate of glycolysis and lactate production over time. In contrast, UMRC6 cells have lower MCT4 expression, and likely slower rate of lactate export. Although UMRC6 cells have higher MCT1 and pyruvate uptake, which may in part explain the higher HP pyruvate to lactate flux, these cells would be less able to maintain a high rate of lactate production over time due to slower lactate export and buildup of intracellular lactate. For that reason, although the pyruvate to lactate flux was lower in the UOK262 cells during the timeframe of the HP MR experiment, the labeled lactate over the 24 hour thermal labeling period was in fact higher in the UOK262 cells, likely as a result of the rapid export of lactate and maintenance of the high glycolytic rate and lactate production in the UOK262 cells.

HP Pyruvate to lactate flux with modulated flow rates in the bioreactor

To demonstrate that UOK262 cells have higher export of lactate, we then compared the HP pyruvate to lactate flux in the UOK262 and UMRC6 cells under different flow rates in the bioreactor. When the flow rate was increased from 2.5ml/min to 3.8ml/min, the observed pyruvate to lactate flux was decreased by 28% in the UOK262 cells, while the flux was not significantly changed in the UMRC6 cells (Fig 4). The observed pyruvate to lactate flux in the bioreactor is affected by 3 factors: first, the transport of pyruvate from the extracellular medium into the intracellular compartment; second, the intracellular enzymatic conversion of pyruvate to lactate mediated by LDH; third, the export of the intracellular lactate to the medium and subsequent removal of the lactate from the NMR-active region. Our data indicated that the HP

pyruvate uptake mediated by MCT1 was not affected by the different flow rates of the bioreactor, as the pyruvate to lactate flux did not significantly change in the UMRC6 cells at the 3 different flow rates. The rate of intracellular enzymatic conversion of pyruvate to lactate is not expected to be altered by the flow rate. In contrast, we noted that the higher flow rate resulted in a decrease in the observed pyruvate to lactate flux in the UOK262 cells, but the higher flow rate did not change the flux in the UMRC6 cells. This indicated that UOK262 cells had higher amount of extracellular lactate, which was readily removed from the NMR-active region by the circulating medium at high flow rate. Such observation supports the notion that UOK262 cells have increased MCT4-mediated export of lactate out of the cells. MCT4 is required for lactate export, pH homeostasis, and the maintenance of the Warburg effect in cancer cells. Our data suggest that MCT4 mediated lactate export may be an important determinant of renal tumor aggressiveness. This is also supported by a recent study which showed that MCT4 protein expression in primary clear cell RCCs was associated with poorer relapse-free survival, and correlated with Fuhrman nuclear grade (6).

Key Research Accomplishments

- Demonstrated that cells derived from the metastatic RCC have increased export of HP lactate to the extracellular space compared to the cells derived from the localized RCC, and that such differences are likely mediated by the differential expression of MCT4. These results suggest that the assessment of lactate production and export using clinically translatable HP probes has the potential to improve the noninvasive characterization of renal tumor aggressiveness.
- Obtained critical preliminary data for metabolic characterization of renal cell carcinomas using hyperpolarized 13C pyruvate MR for future grant applications in this topic.

Reportable Outcomes

1. Abstract presentation:
   Sriram R, Keshari KR, Koelsch B, Van Criekinge M, Kurhanewicz J, **Wang ZJ**. Characterization of Localized and Metastatic Renal Cell Carcinoma Metabolism Using Hyperpolarized and Thermal ^{13}C MR. Presented at International Society of Magnetic Resonance in Medicine (ISMRM) annual meeting 2012, Melbourne, Australia.
2. Manuscript in preparation:
   Hyperpolarized ^{13}C pyruvate metabolism and lactate transport in living human renal cell carcinoma cells
3. Funding received based on work supported by this work:
   - Hyperpolarized ^{13}C MR Markers of Renal Tumor Aggressiveness

Department of Defense Peer Review Cancer Research Program Visionary Postdoctoral Fellowship
PI: Renuka Sriram, PhD
Primary mentor: Zhen Jane Wang, MD

- Hyperpolarized ^{13}C MR Molecular Markers of Renal Tumor Aggressiveness
  University of California Cancer Research Coordinating Committee
  PI: Zhen Jane Wang, MD

Conclusions

In this work, we investigated the real time pyruvate to lactate flux in constantly perfused cells derived from a localized RCC, and from a metastatic RCC using HP ^{13}C pyruvate MR, and interrogated the biochemical basis of the observed HP MR data. We found that a key feature distinguishing between the localized UMRC6 and the metastatic UOK262 RCC cells is the rate of lactate transport outside of the cells. By modulating the flow rates in the bioreactor, we were able to detect a higher rate of lactate export, mediated by the increased MCT4, in the UOK262 cells compared to the UMRC6 cells. From this study, it appears that the rate of lactate export, in addition to the amount of lactate production, may be an important determinant of renal tumor aggressiveness. Such cellular processes can be depicted noninvasively and in real time using HP ^{13}C MR. While our study focused on the investigation of HP ^{13}C pyruvate metabolism and lactate transport in an *ex vivo* system, similar investigations using HP ^{13}C MR can be achieved *in vivo*. For example, it is possible to measure the tumoral extracellular or interstitial pH, which in part reflects the amount of exported lactate, using HP ^{13}C bicarbonate MR (7). Future studies will also be extended to developing diffusion weighted HP MR that can directly measure the amount of intracellular versus exported extracellular lactate.

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Appendicies

Figures and Figure Lengends

Figure. 1. (a) Representative ^{31}P spectra of UMRC6 RCC cells in the bioreactor shows readily visible βNTP resonance (scales to MR visible ATP), which was unchanged during the experiment, indicating metabolic viability of the cells. (b) Peak areas of HP pyruvate and lactate (lactate areas were multiplied by 10) in UMRC6 cells after injection of 5mM of HP pyruvate. (c) The observed HP pyruvate to lactate flux is significantly higher in UMRC6 RCC cells than UOK262 cells (N=4, $p<0.05$).

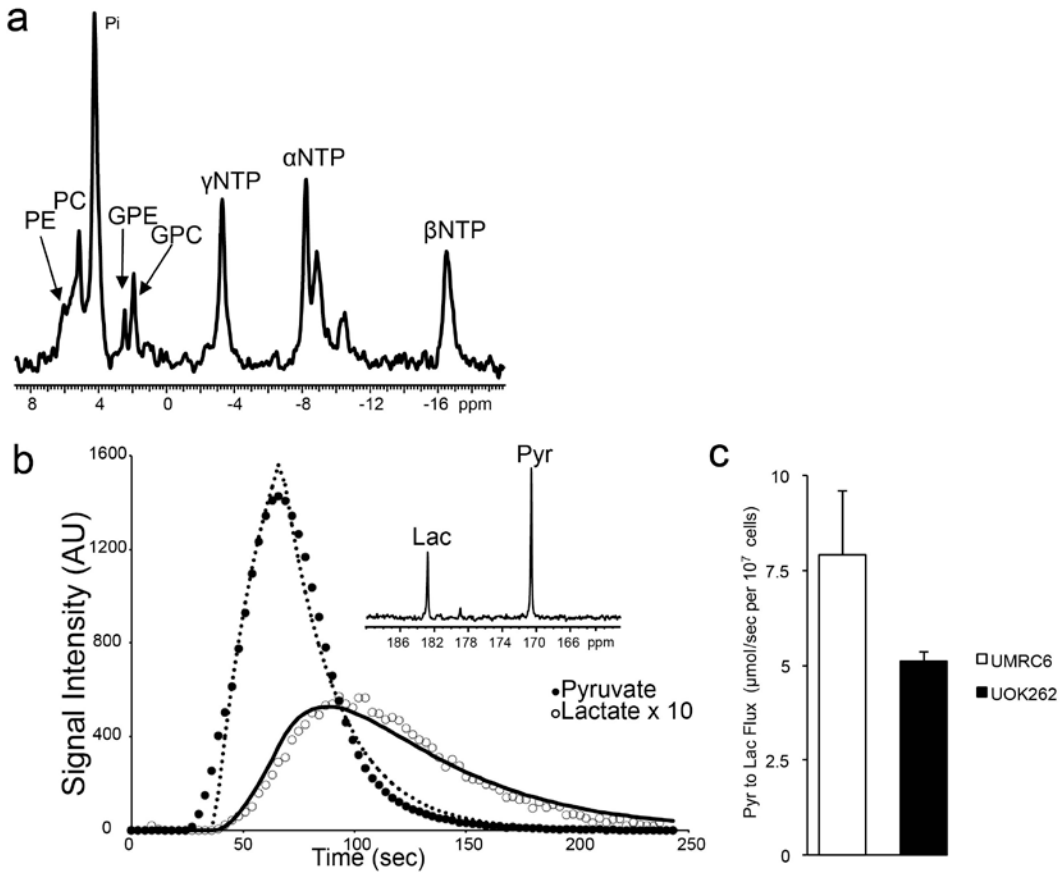


Figure 2. Concentration of total lactate and ^{13}C labeled lactate in the intracellular compartment and the medium of the UMRC6 and UOK262 cells following 24 hour labeling with [3-^{13}C] pyruvate. After 24 hour thermal labeling with pyruvate, the ^{13}C labeled lactate was significantly higher in the UOK262 cells compared to the UMRC6 cells. *: p-value < 0.05.

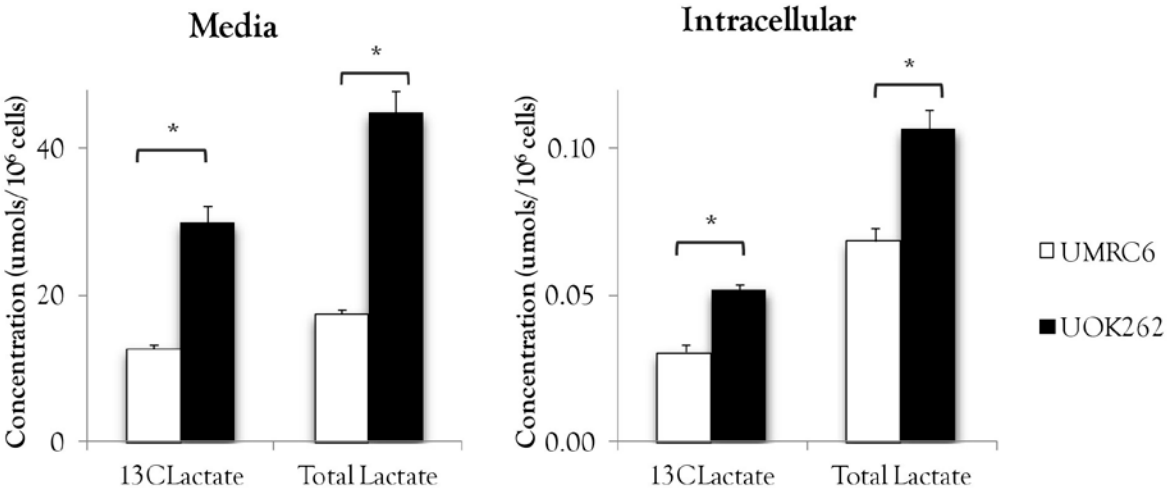


Figure 3. mRNA expression of LDH-A, MCT1, MCT4, and GLUT1 in the UMRC6 and UOK262 cells. LDH: lactate dehydrogenase; MCT: monocarboxylate transporter; GLUT: glucose transporter. MCT1, the predominant transporter for pyruvate into the cells, was approximately 2 fold higher in the UMRC6 cells than the UOK262. MCT4, the predominant transporter of lactate out of the cells, was 75% higher in the UOK262 cells than the UMRC6 cells. LDH-A mRNA expression was significantly higher in the UOK262 cells than the UMRC6 cells. However the LDH activity was not significantly different between the two cell lines (data not shown).

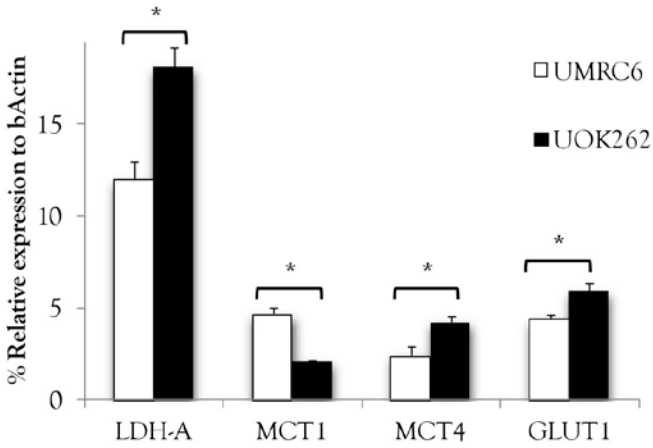
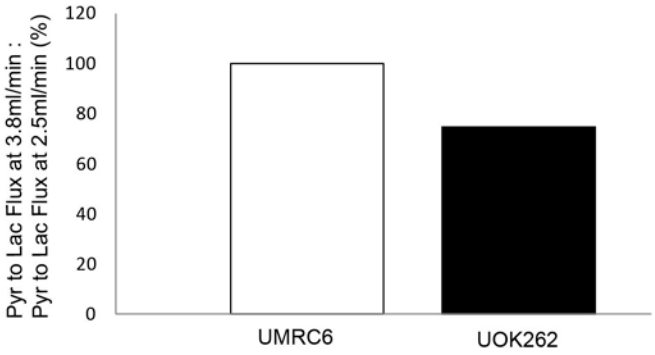


Figure 4. HP pyruvate to lactate flux in the UOK262 and UMRC6 cells under different flow rates in the bioreactor. When the flow rate was increased from 2.5ml/min to 3.8ml/min, the observed pyruvate to lactate flux was decreased by 28% in the UOK262 cells, while the flux was not significantly changed in the UMRC6 cells.



List of personnel receiving pay from the research effort

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